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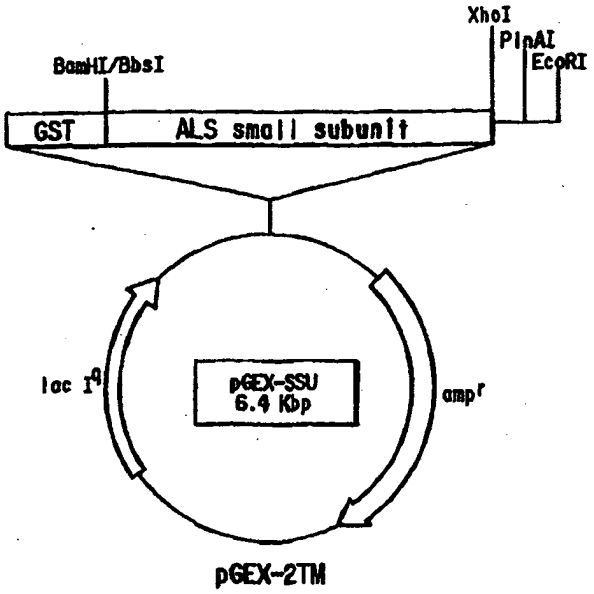
(57) Abstract

The present invention provides a cDNA sequence encoding the small subunit of plant acetolactate synthase ("ALS"). When plant ALS small subunit protein is mixed with the gene product referred to by others as plant acetolactate synthase (and referred to herein as the ALS large subunit), the resulting reconstituted ALS holoenzyme shows both increased catalytic efficiency and solution stability when compared to the large subunit alone. Acetolactate synthase holoenzyme is sensitive to inhibition by herbicidal compounds known to inhibit ALS *in vivo* as their mode of action. Thus, the ALS holoenzyme is useful in screening for novel crop protection chemicals.



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TITLE

USE OF THE SMALL SUBUNIT OF PLANT ACETOLACTATE SYNTHASE FOR NEW HERBICIDE DISCOVERY

FIELD OF INVENTION

This invention is in the field of herbicide discovery. Specifically, this invention pertains to nucleic acid fragments encoding the small subunit of plant acetolactate synthase, and use of the encoded protein to aid in the discovery of new herbicides that inhibit plant acetolactate synthase activity.

BACKGROUND OF THE INVENTION

Acetolactate synthase (ALS; EC 4.1.3.18), also known as acetohydroxy acid synthase, is the first committed step in branched chain amino acid biosynthesis in plants and bacteria. The enzyme is known to be the site of action of the several diverse classes of herbicides including the sulfonylureas [Chaleff, R.S. & Mauvais, C.J., *Science 224*:1443-1445 (1984); LaRossa, R. A. & Schloss, J.V., *J. Biol. Chem. 259*:8753-8757 (1984); Ray, T.B., *Plant Physiol. 75*:827-831 (1984)] and the imidazolinones [Shaner et al., *Plant Physiol. 76*:545-546 (1984)]. Bacterial ALS has been extensively characterized and is known to exist as three isozymes in *E. coli*. Each isozyme is a tetramer composed of two identical large subunits of approximately 60,000 Da molecular weight and two identical smaller subunits ranging in molecular weight between 9,000 Da and 17,000 Da depending on the isozyme of ALS [Chipman et al., in *Biosynthesis of Branched Chain Amino Acids*, eds. Barak, Z., Chipman, D.M. & Schloss, J.V. VCH, Weinheim 243-284 (1990)]. There is a high degree of sequence identity shared among the large subunits in the different isozymes, but very low conservation of sequence is observed when comparing the small subunits. The different isozymes also show various sensitivities to feedback inhibition in the presence of valine, leucine and isoleucine. Only isozyme II is insensitive to feedback inhibition. In the absence of the small subunit, the large subunit is still catalytically active, though at a reduced level and shows no inhibition by effectors such as valine and isoleucine [Weinstock et al., *J. of Bacteriology 174*: 5560-5566 (1992)]. When the large and small subunits of a given isozyme are expressed separately and then mixed, significant increases in specific activity are observed. In the case of isozyme I, a three- to four-fold increase in specific activity is observed upon mixing, whereas in the case of isozyme III, a thirty- to forty-fold increase is observed. Sensitivity to feedback inhibition is restored upon adding the small subunit to the large subunit. Mixing large and small subunits from different isozymes does not produce increases in specific activity or sensitivity to feedback inhibitors.

The enzyme from plants is much less well characterized. Attempts to purify the enzyme from plant extracts have been hampered by the extreme lability of the enzyme and its low abundance. All attempts to purify the enzyme from plant sources have

produced only a single major band on an SDS-PAGE gel which varies in molecular weight from 58,000 Da [Durner, J. & Boger, P. Z. Naturforsch 43c: 850-856 (1988)] to 65,000-66,000 Da [Muhitch et al., *Plant Physiol. 83*: 451-456 (1987)]. Attempts to immunoprecipitate the enzyme from plant extracts also resulted in the isolation of a single band at 65,000-66,000 Da molecular weight [Singh, B.K. et al., *Proc. Natl. Acad. Sci. USA 88*:4572-4576 (1991); Bekkaoui, F. et al., *Physiologia Plantarum 88*:475-484 (1993)]. In the later case, a second band at 36,000 Da was also identified but sequence analysis showed that the protein belonged to a family of aldolases. Thus, since the mid-1980's when the first attempts were made to purify ALS from plant sources, the question as to whether the plant holoenzyme is composed of large and small subunits, in analogy to the bacterial enzyme, has remained unanswered and a matter of some speculation.

SUMMARY OF THE INVENTION

A cDNA clone has now been discovered and identified as the small subunit of plant ALS based upon the sequence identity shared between the peptide encoded by the clone and various bacterial ALS small subunits. A full length clone was obtained from *Nicotiana plumbaginifolia*, expressed in *E. coli*, and partially purified. Mixing the putative small subunit from *Nicotiana* with various large subunits from several sources (*Arabidopsis* and *Nicotiana*) increased the specific activity of the large subunit 4-15 fold. This trend is similar to that observed with the bacterial enzyme and confirms, functionally, the identification of the cDNA clone as the small subunit of plant ALS.

Accordingly, the present invention comprises a nucleic acid fragment encoding the small subunit of plant ALS. The invention also comprises a method for expression and purification of the small subunit, its use in preparing plant ALS holoenzyme and the use of the holoenzyme to screen for potentially herbicidal compounds based upon holoenzyme inhibition.

More specifically, this invention pertains to an isolated nucleic acid fragment encoding the small subunit of a plant acetolactate synthase, the fragment comprising a member selected from the group consisting of (a) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:4; and (b) a nucleotide sequence essentially similar to the nucleotide sequence of (a). Preferred is an isolated nucleic acid fragment wherein the nucleotide sequence is set forth in SEQ ID NO:4.

Another embodiment of the instant invention is a plasmid vector comprising a nucleic acid fragment encoding the small subunit of a plant acetolactate synthase operably linked to at least one suitable regulatory sequence and a transformed host cell comprising the aforementioned plasmid vector.

In yet another embodiment, the instant invention pertains to a method for evaluating at least one compound for its ability to inhibit acetolactate synthase activity, the method comprising the steps of: (a) transforming a host cell with the plasmid vector comprising a nucleic acid fragment encoding the small subunit of a plant acetolactate synthase operably linked to at least one suitable regulatory sequence; (b) facilitating expression of the nucleic acid fragment encoding the small subunit of a plant acetolactate synthase; (c) purifying the small subunit of a plant acetolactate synthase expressed by the transformed host cell; (d) mixing the purified small subunit with the large subunit of a plant acetolactate synthase in a suitable container, thereby forming a plant acetolactate synthase holoenzyme; (e) treating the holoenzyme with a compound to be tested; and (f) comparing the acetolactate synthase activity of the holoenzyme that has been treated with a test compound to the activity of an untreated holoenzyme, thereby selecting compounds with potential for herbicidal activity

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

Figure 1 shows the map of the expression plasmid pTrx-HIS/SSU used to express the *Nicotiana plumbaginifolia* acetolactate synthase small subunit polypeptide.

Figure 2 shows the map of the expression plasmid pGEX-SSU used to express the *Nicotiana plumbaginifolia* acetolactate synthase small subunit polypeptide.

SEQ ID NO:1 represents the nucleotide sequence of the cDNA insert encoding the small subunit of corn acetolactate synthase found in the plasmid clone m15.12.b12.sk20.

SEQ ID NO:2 is the sequence of oligodeoxynucleotide primer SU5R used to prime first strand cDNA synthesis of *Nicotiana plumbaginifolia* ALS small subunit.

SEQ ID NO:3 is the sequence of oligodeoxynucleotide primer SU4R used for PCR amplification of the single stranded cDNA representing *Nicotiana plumbaginifolia* ALS small subunit.

SEQ ID NO:4 is the full-length cDNA sequence of the *Nicotiana plumbaginifolia* ALS small subunit contained in the plasmid pSSU.NP1.

SEQ ID NO:5 and SEQ ID NO:6 are oligodeoxynucleotides (pTrx linker1 and pTrx linker2, respectively) that were used to aid construction of the plasmid vector pTrx-Bst1107.

SEQ ID NO:7 and SEQ ID NO:8 are oligodeoxynucleotides (HIS-TAG5 and HIS-TAG3, respectively) that were used to aid construction of the plasmid vector pTrx-HIS.

SEQ ID NO:9 and SEQ ID NO:10 are PCR primers (SSU-PCR1 and SSU-PCR2, respectively) used for amplification of the insert in cDNA clone SSU.NP1.

SEQ ID NO:11 and SEQ ID NO:12 are oligonucleotides (CAM19 and CAM20, respectively) used to modify the plasmid vector pGEX-2T to create the plasmid vector pGEX-2TM.

SEQ ID NO:13 and SEQ ID NO:14 are oligodeoxynucleotides (SSU oligo 9 and SSU oligo 10, respectively) that were used to aid construction of the plasmid vector pGEX-SSU.

SEQ ID NO:15 and SEQ ID NO:16 are oligodeoxynucleotides (SSU oligo 5 and SSU oligo 6, respectively) that were used to aid construction of the plasmid vector pGEX-SSU.

SEQ ID NO:17 and SEQ ID NO:18 are oligodeoxynucleotides (mt704+ and mt800-, respectively) that were used to aid construction of the plasmid vector pMTDRALS.

SEQ ID NO:19 is the full-length cDNA sequence of the *Nicotiana plumbaginifolia* ALS large subunit contained in the plasmid pALS10.

BIOLOGICAL DEPOSITS

The following plasmid has been deposited under the terms of the Budapest Treaty at American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, and bears the following accession number:

Plasmid	Accession Number	Date of Deposit
pSSU.NP1	ATCC 97876	February 12, 1997

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a cDNA sequence encoding the small subunit of plant acetolactate synthase ("ALS"). When plant ALS small subunit protein ("SSU") is mixed with the gene product referred to by others as plant acetolactate synthase (and referred to herein as the ALS large subunit or "LSU"), the resulting reconstituted ALS holoenzyme shows both increased catalytic efficiency and solution stability when compared to the large subunit alone. The beneficial effects of reconstituting native ALS with this small subunit is not species-specific as shown by the ability of the *N. plumbaginifolia* small subunit to form an active complex with large subunits from other plant species.

It is expected that divergent plant species will have ALS small subunit genes that encode the same functional protein as that encoded by the cDNA clone described herein. Therefore, it is expected that the invention can also be accomplished using ALS small subunit sequences from other plant species, both monocotyledonous and dicotyledonous. Indeed, based upon the work presented here, it is expected that the invention may be accomplished by mixing small and large ALS subunits from divergent species.

To accomplish the invention, single pass DNA sequence analysis was performed on individual clones from a cDNA library made using RNA from corn embryos that were harvested 15 days post pollination. The sequences were compared to known

sequences in the GenBank database until a clone was identified as the small subunit of plant ALS by the similarity of its coding region with those of various bacterial ALS small subunits. A DNA fragment comprising the coding region from this clone was isolated and operably linked to suitable bacterial regulatory sequences to create plasmids capable of directing the expression of plant ALS small subunit in *E. coli* as thioredoxin (TRX) and glutathione-S-transferase (GST) fusion proteins. These plasmids were introduced separately into suitable strains of *E. coli* and the TRX- and GST-plant SSU fusion proteins were produced after suitable induction and expression of the chimeric genes encoding the fusion proteins.

The recombinant ALS large and small subunits may be produced using any number of methods by those skilled in the art. Such methods include, but are not limited to, expression in bacteria, eukaryotic cell culture, *in planta*, and using viral expression systems in suitably infected organisms or cell lines. Large and small ALS subunits may be expressed separately as mature proteins, or may be co-expressed in *E. coli* or another suitable expression background. In addition, whether expressed separately or in combination, large and small subunits may be expressed either as mature forms of the proteins as observed *in vivo* or as fusion proteins by covalent attachment to a variety of enzymes, proteins or affinity tags. Common fusion protein partners include glutathione S-transferase ("GST"), thioredoxin ("Trx"), maltose binding protein, and C- and/or N-terminal hexahistidine polypeptide ("$(His)_6$"). The fusion proteins may be engineered with a protease recognition site at the fusion point so that fusion partners can be separated by protease digestion to yield intact mature large or small subunit. Examples of such proteases include thrombin, enterokinase and factor Xa. However, any protease can be used which specifically cleaves the peptide connecting the fusion protein and either the large or small subunit.

Purification of the ALS subunits or the holoenzyme may utilize any number of separation technologies familiar to those skilled in the art of protein purification. Examples of such methods include, but are not limited to, homogenization, filtration, centrifugation, heat denaturation, ammonium sulfate precipitation, desalting, pH precipitation, ion exchange chromatography, hydrophobic interaction chromatography and affinity chromatography, wherein the affinity ligand represents a substrate, substrate analog or inhibitor. When the ALS subunits are expressed as fusion proteins, the purification protocol may include the use of an affinity resin which is specific for the fusion protein tag attached to the expressed large or small subunit or an affinity resin containing ligands which are specific for the small subunit. In a preferred embodiment of the invention, small subunit of ALS is expressed as a fusion protein coupled to the C-terminus of thioredoxin. In addition, a $(His)_6$ peptide may be engineered into the N-terminus of the fused thioredoxin moiety to afford additional opportunities for

affinity purification. The preferred purification protocol embodied in the examples involves the use of arsene oxide affinity chromatography with the commercially available ThioBond™ resin (Invitrogen Corporation, San Diego, CA) which has affinity for thioredoxin. Other suitable affinity resins could be synthesized by linking the appropriate ligands to any suitable resin such as Sepharose-4B. In a preferred embodiment of the invention, the thioredoxin-SSU fusion protein is eluted using dithiothreitol; however, elution may be accomplished using other reagents which interact to displace the thioredoxin from the resin. These reagents include β–mercaptoethanol or other reduced thiol. The eluted fusion protein may be subjected to further purification by traditional means as stated above, if desired. Proteolytic cleavage of the thioredoxin fusion protein and the ALS small subunit to yield a complete small subunit may be accomplished after the fusion protein is purified, while the protein is still bound to the ThioBond™ affinity resin or other resin, or in the presence of an ALS large subunit.

The generality of this procedure is demonstrated in a preferred embodiment of the invention whereby the small subunit protein is expressed and purified as a fusion to GST. The preferred purification protocol for this fusion protein embodied in the examples involves the use of a glutathione-containing Sepharose- or agarose-based affinity resin which has affinity for GST. Other suitable affinity resins could be synthesized by linking the appropriate ligand to a suitable resin. Moreover, other purification protocols could be developed using conventional chromatography or other protein purification techniques. In a preferred embodiment of the invention, the GST-SSU is eluted using glutathione at pH 9.0; however, elution may be accomplished using other reagents and conditions of pH and ionic strength which serve to weaken the interaction between GST and the glutathione-containing resin.

In a preferred embodiment of the invention, the *Arabidopsis* ALS large subunit is expressed and purified as a fusion protein with glutathione-S-transferase; however, large subunit may be purified by a number of different means as stated above.

Another preferred embodiment of the invention comprises expression of the *Nicotiana plumbaginifolia* ALS-LSU as a mature enzyme containing a partial chloroplast transit peptide. As exemplified herein, this enzyme is purified using conventional protein purification procedures. Variations on these procedures using different column materials and conditions can easily be envisioned by the skilled artisan. This enzyme may also be expressed as a fusion protein and affinity-purified as described above.

Acetolactate synthase holoenzyme, which is defined as a combination of large and small subunits, may be prepared by (i) mixing partially or completely purified large and small subunits, (ii) co-purification of the holoenzyme which is either prepared by co-

expression or by mixing cell extracts containing individually expressed subunits either as fusion proteins or as mature subunits, or (iii) by mixing purified or partially purified fusion proteins. In the example provided of the invention, the fusion protein of the small subunit is mixed either with a fusion protein of the large subunit or a mature form of the large subunit. In both cases, increases in specific activity were observed upon incubation compared to the large subunit by itself. Activity is further enhanced by the addition of a specific protease, in this case thrombin, to cleave the fusion protein affinity tag to produce mature small and in some cases large subunits. The inclusion of such non-specific proteins such as bovine serum albumin did not produce the same effect as the presence of the small subunit. In the present example, phosphate buffered saline at pH 8 was used. Buffers in the range of pH 6.5 to 9 are preferred with buffers in the range of pH 7 to 7.6 being optimal. Cofactors required for ALS activity may also be present in the holoenzyme reconstitution such as flavin adenine dinucleotide, thiamine pyrophosphate and divalent metal ions. Stabilizing agents such as dithiothreitol and glycerol may also be used.

Acetolactate synthase holoenzyme is sensitive to inhibition by herbicidal compounds known to inhibit ALS *in vivo* as their mode of action. Thus, the ALS holoenzyme is useful in screening for novel crop protection chemicals.

DEFINITIONS

In the context of this disclosure, a number of terms shall be utilized. As used herein, the term "nucleic acid" refers to a large molecule which can be single-stranded or double-stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. A "nucleic acid fragment" is a fraction of a given nucleic acid molecule. In higher plants, deoxyribonucleic acid (DNA) is the genetic material while ribonucleic acid (RNA) is involved in the transfer of the information in DNA into proteins. A "genome" is the entire body of genetic material contained in each cell of an organism. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers.

As used herein, "essentially similar" refers to DNA sequences that may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is therefore understood that the invention encompasses more than the specific exemplary sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce silent changes that do not substantially affect the functional properties of the resulting protein molecule are also contemplated. For example, alteration in the gene

sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated; thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Moreover, the skilled artisan recognizes that "essentially similar" sequences encompassed by this invention are also defined by their ability to hybridize, under relatively stringent conditions (2X SSC, 0.1% SDS, 55°C), with the sequences exemplified herein.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. "Native" gene refers to the gene as found in nature with its own regulatory sequences. "Chimeric" gene refers to a gene comprising heterogeneous regulatory and coding sequences. "Endogenous" gene refers to the native gene nominally found in its natural location in the genome. A "foreign" gene refers to a gene not nominally found in the host organism but that is introduced by gene transfer.

"Coding sequence" refers to a DNA sequence that codes for a specific protein and excludes the non-coding sequences.

"Initiation codon" and "termination codon" refer to a unit of three adjacent nucleotides in a coding sequence that specifies initiation and chain termination, respectively, of protein synthesis (mRNA translation). "Open reading frame" refers to the amino acid sequence encoded between translation initiation and termination codons of a coding sequence.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from post-transcriptional processing of the primary transcript. "Messenger" RNA (mRNA) refers to RNA that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA, one strand of which is complementary

to and derived from mRNA by reverse transcription. "Sense" RNA refers to RNA transcript that includes the mRNA.

As used herein, "regulatory sequences" refer to nucleotide sequences located upstream (5'), within, and/or downstream (3') to a coding sequence, which control the transcription and/or expression of the coding sequences, potentially in conjunction with the protein biosynthetic apparatus of the cell. These regulatory sequences include promoters, translation-leader sequences, transcription-termination sequences, and polyadenylylation sequences.

"Promoter" refers to a DNA sequence in a gene, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions. It may also contain enhancer elements.

The term "operably linked" refers to nucleic acid sequences on a single nucleic acid molecule which are associated so that the function of one is affected by the other. For example, a promoter is operably linked with a structural gene (i.e., a gene encoding aspartokinase that is lysine-insensitive as given herein) when it is capable of affecting the expression of that structural gene (i.e., that the structural gene is under the transcriptional control of the promoter).

The term "expression", as used herein, is intended to mean the production of the protein product encoded by a gene. More particularly, "expression" refers to the transcription and stable accumulation of the sense (mRNA) or antisense RNA derived from the nucleic acid fragment(s) of the invention that, in conjunction with the protein apparatus of the cell, results in the accumulation of the encoded protein product. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. "Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

The "3' non-coding sequences" refers to the DNA sequence portion of a gene that contains a polyadenylation signal and any other regulatory signal capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

The "translation leader sequence" refers to that DNA sequence portion of a gene between the promoter and coding sequence that is transcribed into RNA and is present in the fully processed mRNA upstream (5') of the translation start codon. The

translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency.

"Mature" protein refers to a post-translationally processed polypeptide wherein a number of amino acids at the N- and/or C-terminus of the primary translation product has been removed by proteolysis. "Precursor" protein refers to the primary product of translation of mRNA. A "chloroplast targeting signal" is an amino acid sequence which is translated in conjunction with a protein and directs it to the chloroplast. "Chloroplast transit sequence" refers to a nucleotide sequence that encodes a chloroplast targeting signal.

"Fusion protein" refers to a polypeptide that is produced with additional amino acids at either its N-or C-terminus to aid in its expression and/or purification.

"Transformation" herein refers to the transfer of a foreign gene into a host organism and its genetically stable inheritance. "Host cell" means the cell that is transformed with the introduced genetic material.

"Inhibition" refers to a decrease in the catalytic activity of an enzyme or holoenzyme complex by reversible or irreversible binding of a chemical to the enzyme.

"Holoenzyme" is defined as an intact enzyme containing all of the subunits and cofactors required for full activity. In the case of a plant acetolactate synthase these include a small subunit comprising a protein of approximately 45,000 Da, a large subunit comprising a protein of approximately 65,000 Da, and cofactors flavin adenine dinucleotide, thiamine pyrophosphate and divalent metal ions.

"Specific activity" is the number of enzyme units per mg protein. "Units" is defined as the micromoles of product produced per minute of reaction time.

EXAMPLES

The present invention is further defined in the following examples. It will be understood that the examples are illustrative only and the present invention is not limited to uses described in the examples. From the above discussion and the following examples, one skilled in the art can ascertain, and without departing from the spirit and scope thereof, can make various changes and modifications to the invention to adapt it to various uses and conditions. All such modifications are intended to fall within the scope of the claims.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter "Sambrook").

EXAMPLE 1

Isolation of the cDNA for the small subunit of acetolactate synthase

Embryos were harvested from ears of field grown LH195 corn 15 days post pollination and quickly frozen in liquid nitrogen. Total RNA was prepared from frozen tissue using guanidinium thiocyanate extraction and CsCl purification as described by Colbert et al. [*Proc. Natl. Acad. Sci. USA 45* 1703-1708 (1983)]. Total RNA was sent to Stratagene Cloning Systems (La Jolla, CA) where a directional cDNA library was made in the vector lambda Uni-ZAP™ using their custom cDNA library service. The library was plated at low density and individual plaques were randomly selected and picked into SM (50 mM Tris-HCl pH 7.5, 10 mM $MgSO_4$, $7H_2O$, 10 mM NaCl, 0.01% gelatin). After elution of the phage from the agar plug, the resulting phage stocks were diluted 1:10, and cDNA insert was amplified from the phage using the polymerase chain reaction (PCR) using vector-directed primers (T3/T7). PCR products were purified using Qiagen PCR purification kit and an aliquot of the purified PCR product was checked by agarose gel electrophoresis for DNA integrity. The DNA was then sequenced using an Applied Biosystems Inc. (Foster City, CA) Model 373 DNA sequencer and ABI dye terminator sequencing kit. The resulting single pass cDNA sequence was compared to sequences in GenBank using NCBI Blast facility, and in this way, a clone designated m15.12.b12.sk20 was identified by the sequence similarity of its coding region to various bacterial small subunit of acetolactate synthase (SEQ ID NO:1).

Nicotiana plumbaginifolia was grown to the 5-leaf stage in Metromix 350 at 26°C in a growth chamber maintained at 75% relative humidity using a 16 hr/8 hr day/night cycle. Plants were removed from soil with roots, rinsed in 0.5 X Hoagland's solution to remove as much adhering soil as possible and frozen in liquid nitrogen. Total RNA was prepared from frozen tissue using guanidinium thiocyanate extraction and CsCl purification as described by Colbert et al. [*Proc. Natl. Acad. Sci. USA 45* 1703-1708 (1983)]. Total RNA was sent to Clontech Laboratories. Inc. (Palo Alto, CA) where a directional cDNA library in the vector lambda ZAP II™ was made using their custom cDNA library service.

A total of $3x10^6$ phage were plated on 25x25 cm square NZY plates (10 g NZ amine, 5 g yeast extract, 2 g $MgSO_4$) at a density of $2.5x10^5$ plaques/plate. To do this, $2.5x10^5$ phage were mixed with 5 mL of an overnight culture of *E. coli* XL-1 Blue grown in NZY containing 0.2% maltose and cells were incubated at 37°C for 15 min. The infected culture was mixed with 40 mL of NZY top agar (ZY media containing 0.7% agarose) and pored onto the plate. After the top agarose hardened, plates were incubated at 37°C for 6-8 h and then stored at 4°C. Phage lifts were then performed by layering dry MagnaGraph™ nylon transfer membranes (Micron Separations Inc.,

Westborough, MA) on top of the phage plates for 5 min. Membranes were transferred with their DNA side facing upward onto Whatman 3MM paper saturated with 0.5 M NaOH, 1.5 M NaCl. After 5 min, membranes were transferred onto Whatman 3MM paper saturated with 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl and incubated for an additional 5 min. Membranes were then rinsed with 2X SSPE (20X SSPE is 3 M NaCl, 0.1 M Na_2HPO_4, 20 mM EDTA, pH 7.4), air dried for 10 min and DNA was crosslinked to the membrane using a Stratalinker™ UV Crosslinker (Stratagene; La Jolla, CA) in the "Auto Cross Link" mode by following the manufacturer's protocol. Membranes were stored at 4°C in sealed polyethylene bags.

The cDNA insert from 25 μg of the m15.12.b12.sk20 plasmid was digested with Eco RI and Xho I and the digestion products were separated by electrophoresis using a 7.5% polyacrylamide gel. The gel was stained with ethidium bromide and the 750 bp cDNA fragment was recovered as described [*Methods in Enzymology, Vol. 65* 499-560, Academic Press, New York (1980)]. The DNA was labeled with ^{32}P by random priming using a RadPrime™ Labeling System kit (GibcoBRL, Gaithersburg, MD) to a specific activity of $>10^9$ dpm/μg as per the manufacturer's protocol. Following removal of unincorporated label by two precipitations of the DNA with ethanol, the labeled DNA was denatured by boiling for 5 min and quench cooling to 0°C on ice.

Phage lift membranes were prehybridized for 4 h at 65°C in 6X SSPE, 0.5% SDS, 1 mM EDTA, 5X Denhardt's solution, 100 μg denatured and sonicated calf thymus DNA/mL. Membranes were then hybridized overnight with the m15.12.b12.sk20 probe in 6X SSPE, 0.5% SDS, 1 mM EDTA, 2X Denhardt's solution, 100 μg denatured and sonicated calf thymus DNA/mL. The membranes were washed twice at room temperature with 2X SSPE, 0.1% SDS, twice with 2X SSPE, 0.1% SDS at 55°C, air dried and exposed to Kodak X-OMAT XAR-5 film overnight at -80°C using a single intensifying screen. Agarose plugs containing plaques that hybridize to the probe were picked from appropriate plates and phage were eluted into 1 mL of SM (50 mM Tris-HCl pH 7.5, 0.1 M NaCl, 10 mM $MgSO_4$, 0.01% gelatin) by overnight incubation at 4°C. Plaque purification of phage was performed by serially diluting eluted phage with SM, infecting 100 uL cultures of *E. coli* XL-1 Blue with 100 uL aliquots of the dilutions and growing the infected bacteria overnight at 37°C on NZY plates. Lifts of these plates were prepared and hybridized with labeled insert cDNA from the m15.12.b12.sk20 plasmid as described above. Hybridizing plaques were repeatedly subjected to this procedure until all plaques on a given plate hybridized with the probe DNA. In this manner, 12 phage isolates were prepared and stored at 4°C in 0.5 mL of SM containing 50 uL of $CHCl_3$.

Plasmid DNA was obtained from each pure phage isolate as follows. Overnight cultures of *E. coli* XL-1 Blue and *E. coli* SOLR were grown up in NZY and LB media,

respectively. XL-1 Blue cells were diluted to an A_{600} of 1.0 with NZY and 200 uL of this dilution was infected with 100 uL of pure phage stock and 1 uL of a 10^{10} pfu/mL stock of ExAssist™ helper phage (Stratagene). Following a 15 min incubation at 37°C, cells were diluted with 3 mL of 2X YT media and incubated with shaking at 37°C for 2 hr. The culture was heated to 70°C for 20 min and then centrifuged at 10,000Xg for 5 min at 4°C. The supernatant was transferred to a fresh tube and 2 uL of this supernatant was mixed with 200 uL of SOLR cells from the overnight culture. Following a 15 min incubation at 37°C, 10 and 100 uL aliquots of the cultures were spread onto LB plates supplemented with 100 μg ampicillin/mL and the plates were incubated at 37°C overnight. Plasmids from individual antibiotic resistant colonies were analyzed for cDNA inserts by digestion with Eco RI and Xho I.

Sequence analyses were performed on plasmids harboring the largest sized inserts by the dideoxy sequencing method using a Sequenase™ DNA Sequencing Kit (Stratagene, La Jolla, CA). The 5' end of the ALS small subunit mRNA was confirmed by rapid amplification of cDNA ends (5' RACE) using a 5'/3' RACE kit form Boehringer Mannheim (Indianapolis, IN). To this end total RNA prepared as described above was used as the target for RACE following the manufacturer's recommendation using oligodeoxynucleotide SU5R of the sequence

SU5R 5'-CCCAGTACGAGCAATTTCTC-3' (SEQ ID NO:2)

to prime first strand cDNA synthesis. After dA tailing of the ss cDNA, 35 cycles of PCR was performed using the oligo dT anchor primer supplied with the 5'/3' RACE kit and the specific ALS SSU primer SU4R

SU4R 5'-GTGGCTCCTTGGATAGATCT-3' (SEQ ID NO:3)

using a temperature profile of: 95°C for 1 min, 54°C for 1 min, 72°C for 1 min. The RACE products were separated by electrophoresis in a 2% agarose gel. A region of the gel containing 400-500 bp DNA fragments was excised and the DNA is purified using a QIAquick™ gel extraction kit (Qiagen, Inc., Chatsworth, CA). The purified RACE product was ligated into the vector pGEM-T (Promega Corp., Madison, WI) using conditions described earlier, 1 uL of the ligation reaction was used to transform competent *E. coli* JM109. Aliquots of the transformation mixture were spread on LB plates containing 100 μg ampicillin/mL and the plates were incubated at 37°C overnight. Plasmids from individual antibiotic resistant colonies were analyzed for cDNA inserts by digestion with Nar I and Nco I. The inserts of clones showing the largest inserts were subjected to DNA sequence analysis as described above and the resulting RACE sequence was combined with that obtained from clones isolated from

screening of a lambda ZAP II™ cDNA library to assemble the full-length sequence of the *N. plumbaginifolia* small subunit of acetolactate synthase cDNA. This clone was renamed pSSU.NP1, and is comprised of the sequence presented as SEQ ID NO:4 contained within the plasmid pBluescript(SK-).

EXAMPLE 2

Construction of acetolactate synthase small subunit expression vectors

Construction of pTrx-Bst1107

Five micrograms of oligodeoxynucleotides pTrx linker1 and pTrx linker2 were phosphorylated separately for 30 min at 37°C in 100 uL of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 100 units of T_4 polynucleotide kinase. 3.2 uL aliquots of each phosphorylated primer were then combined, diluted to 50 uL with H_2O and heated to 70°C for 20 min and allowed to cool to ambient temperature.

pTrx linker1 5'-GTATACGAGGATCCTCTAGAG-3' (SEQ ID NO:5)

pTrx linker2 5'-TCGACTCTAGAGAGTCCTCGTATAC-3' (SEQ ID NO:6)

The bacterial expression vector pTrxFUS (Invitrogen) was digested to completion with Kpn I and the resulting 3' overhang was removed by incubation of the DNA with the Klenow fragment of DNA polymerase I for 1 h at 25°C in a buffer consisting of 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM each of dATP, dCTP, dGTP, and dTTP, and 1 unit of Klenow/μg of DNA. The DNA was then extracted sequentially with equal volumes of phenol:$CHCl_3$:isoamyl alcohol (25:24:1) and $CHCl_3$ and precipitated on dry ice for 20 min after adding 0.1 volume of 3 M sodium acetate pH 6 and 2 volumes of ethanol. DNA was recovered by centrifugation at 14,000 X g for 10 min. The DNA was digested to completion with Sal I and dephosphorylated with calf intestinal alkaline phosphatase. One μg of this DNA was then ligated with 1 uL of the dilute mixture of phosphorylated pTrx linker1 and pTrx linker2 prepared above at 16°C for 3 h in 10 uL of 66 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM dithioerythritol, and 5 units of T_4 DNA ligase. The ligation reaction was diluted to 50 uL with H_2O and 2 uL of this dilution was used to transform competent Max Efficiency *E. coli* HB101 (GibcoBRL, Gaithersburg, MD) using the protocol supplied with the cells. Aliquots of the transformation mixture were spread on LB plates containing 100 μg/mL ampicillin and the plates were incubated at 37°C overnight. Plasmids from individual antibiotic resistant colonies were analyzed for incorporation of the desired double-stranded oligonucleotide by dideoxy sequence analysis using the commercially available forward

and reverse sequencing primers (Invitrogen). One clone found to contain the correct insert is designated pTrx-Bst1107.

Construction of pTrx-HIS

A polymerase chain reaction is performed using plasmid pTrxFUS as a target and the oligodeoxynucleotides HIS-TAG5 and HIS-TAG3 as primers.

HIS-TAG5 5'-GGAATTCTCCATATGCACCATCATCATCATCATAGCGATAAAAT TATTCAC-3' (SEQ ID NO:7)

HIS-TAG3 5'-CCTGTACGATTACTGCAGGTC-3' (SEQ ID NO:8)

The reaction mixture was assembled in a total volume of 500 uL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 800 ng plasmid pTrxFUS, 150 pmoles each of HIS-TAG5 and HIS-TAG3, and 12.5 units of AmpliTaq polymerase (Perkin Elmer Cetus, Norwalk, CT). The mixture was divided into 5 100 µL aliquots and the five tubes were subjected to 18 cycles of a temperature profile of: 95°C for 1 min, 55°C for 1 min, 72°C for 1 min. A final cycle of 95°C for 1 min, 55°C for 1 min, 72°C for 5 min completed the reaction. The five aliquots were pooled and extracted sequentially with equal volumes of phenol:$CHCl_3$:isoamyl alcohol (1:1:1) and $CHCl_3$. The DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate pH 6 and 2 volumes of ethanol followed by a 20 min incubation on dry ice. DNA was recovered by centrifugation at 14,000 x g for 10 min, dried *in vacuo* and dissolved in 100 uL of 10 mM Tris-HCl pH 7.5, 1 mM EDTA (TE).

One half of the PCR product was digested to completion with Nde I and Rsr II. The digest was extracted sequentially with equal volumes of phenol:$CHCl_3$:isoamyl alcohol (25:24:1) and $CHCl_3$. The DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate pH 6 and 2 volumes of ethanol followed by 20 min on dry ice. DNA was recovered by centrifugation at 14,000 X g for 10 min and the pellet was dried *in vacuo* and dissolved in 15 uL of TE. A 120 ng aliquot of Nde I/Rsr II digested PCR product was ligated with 1 µg of the vector pTrx-Bst1107 that had been digested to completion with Nde I and Rsr II and dephosphorylated with calf intestinal alkaline phosphatase in a 10 uL ligation reaction using conditions described above.

Competent *E. coli* GI 724 cells were prepared by following the protocol supplied by Invitrogen. A 4 uL aliquot of the ligation mixture was used to transform 110 uL of competent *E. coli* GI 724 cells using the chemical transformation protocol supplied by Invitrogen, Inc. Aliquots of the transformation mixture were spread on RMG plates [6 g/L Na_2HPO_4, 3 g/L K_2HPO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 2% amicase (acid casein hydrolysate; Sigma Chemical Co., St. Louis, MO), 1 mM $MgCl_2$, pH 7.4, 0.5% glucose,

12.5% agar] containing 100 μg/mL ampicillin and the plates were incubated at 30°C overnight. Plasmids from individual antibiotic resistant colonies were analyzed for inserts by dideoxy sequencing until a colony is found to containing the expected PCR product encoding a $(His)_6$ incorporated into the Nde I/Rsr II site of pTrx-Bst1107. This plasmid was designated pTrx-HIS.

Construction of pTrx-HIS/SSU

A polymerase chain reaction was performed using cDNA clone SSU.NP1 as a target and the oligodeoxynucleotides SSU-PCR1 and SSU-PCR3 as primers.

SSU-PCR1

5′-AACAACAACGATATCAGACAAAAGACTAGGCGCCA-3′ (SEQ ID NO:9)

SSU-PCR3

5′-AACAACGGATCCAACCAACTTATATAGTTGCTGCACCA-3′ (SEQ ID NO:10)

PCR was performed using the conditions described above with 150 pmoles each of SSU-PCR1 and SSU-PCR3, and 800 ng of SSU.NP1 in the reaction. The PCR reaction was then extracted, precipitated, and redissolved in 100 uL of TE as described earlier.

One half of the PCR product was digested to completion with Eco RV and Bam HI. The digest was then extracted sequentially with equal volumes of phenol:$CHCl_3$:isoamyl alcohol (1:1:1) and $CHCl_3$. The DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate pH 6 and 2 volumes of ethanol followed by 20 min on dry ice. DNA was recovered by centrifugation at 14,000 x g for 10 min. The pellet was dried *in vacuo* and dissolved in 15 uL of TE. Approximately 250 ng of Eco RV/Bam HI digested PCR product was ligated with 1 μg of the vector pTrx-HIS that had been digested to completion with Bst1107I and Bam HI and dephosphorylated with calf intestinal alkaline phosphatase in a volume of 10 uL. A 4 uL aliquot of the ligation mixture was used to transform competent *E. coli* GI724 cells as described above and aliquots of the transformation mixture were spread on RMG plates and incubated at 30°C overnight. Plasmids from individual antibiotic resistant colonies were analyzed for inserts by digestion with Nde I and Xba I until a colony was found that contains a plasmid showing a 1.7 kbp Nde I/Xba I insert. This plasmid was designated pTrx-HIS/SSU.

The same Eco RV/Bam HI digested PCR product was ligated with 1 μg of pTrx-Bst1107 that had been previously digested with Bst1107 and BamHI to yield the plasmid pTrx-BST/SSU.

Construction of pGEX-2TM

Additional restriction sites were added to the multiple cloning site of protein expression vector pGEX-2T (Pharmacia Biotech, Uppsala, Sweden). pGEX-2T (10 μg)

was digested to completion with BamH I and EcoRI and the digestion products were separated by electrophoresis using a 1% agarose gel. The 4.95 kbp band was excised from the gel and the DNA was recovered using a QIAquick™ Gel Extraction Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Sodium acetate (pH 5.2) was added to the final column eluant to give a concentration of 0.3 M; 10 µg of tRNA and 2 volumes of cold ethanol were then added and the DNA was recovered by centrifugation. The pellet was washed with 70% ethanol, air dried at room temp, dissolved in 10 µL TE and quantified by running a 1 µL aliquot on a 1% agarose gel and comparing the band intensity with that of a commercial mass ladder standard.

Approximately 50 ng of digested plasmid were mixed with 70 ng each of phosphorylated CAM19 and CAM20 oligonucleotides and the mixture was incubated at 45°C for 5 minutes and cooled on ice. The ligated oligonucleotides create the following sites: BamH I, NcoI, SalI, XhoI, PinAI and EcoRI.

CAM19 5'-GATCCATGGTCGACTCGAGACCGGTG-3' (SEQ ID NO:11)

CAM20 5'-AATTCACCGGTCTCGAGTCGACCATG-3' (SEQ ID NO:12)

T4 DNA ligase buffer and 1 unit of T4 DNA ligase (GibcoBRL) were added and the mixture was incubated at 37° C for 1 hour. The ligation mix was then transformed into DH5α Max Efficiency Competent *E. coli* (GibcoBRL) using a standard heat shock protocol (Sambrook) and spread onto LB plates containing 100 µg carbenicillin/mL. The plates were incubated overnight at 37°C and plasmids were isolated from carbenicillin-resistant bacterial colonies and analyzed for the presence of both PinAI and NcoI restriction sites by restriction endonuclease digestion. A plasmid with PinAI and NcoI sites was selected and designated pGEX-2TM.

Construction of pGEX-SSU

Five micrograms of oligodeoxynucleotides SSU oligo 9 and SSU oligo 10 were phosphorylated separately for 30 min at 37°C in 100 µL of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 100 units of T_4 polynucleotide kinase.

SSU oligo 9 5'-GATCTGAAGACAACATGATCAGACAAAAGACTAGGCGCC
AACAACAAGGATCC -3' (SEQ ID NO:13)

SSU oligo 10 5'-TCGAGGATCCTTGTTGTTGGCGCCTAGTCTTTTGTCTGA
TCATGTTGTCTTCA -3' (SEQ ID NO:14)

Four µL aliquots of each phosphorylated primer were combined, diluted to 40 µL with H_2O, heated to 70°C for 20 min and allowed to cool to ambient temperature.

The bacterial plasmid vector pBluescript SK(+) (Stratagene, La Jolla, CA) was digested to completion with Bam HI and Xho I dephosphorylated with calf intestinal alkaline phosphatase. One μg of this DNA was then ligated with 1 μL of the mixture of phosphorylated SSU oligo 9 and SSU oligo 10 at 16°C for 3 h in 10 μL of 66 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM dithioerythritol using 5 units of T_4 DNA ligase. The ligation reaction was diluted to 50 μL with H_2O and 2 μL was used to transform competent Max Efficiency *E. coli* HB101 (Gibco/BRL, Gaithersburg, MD) using the protocol supplied with the cells. Aliquots of the transformation mixture were spread on LB plates containing 100 μg/mL ampicillin and the plates were incubated at 37°C overnight. Plasmids from individual antibiotic resistant colonies were analyzed for incorporation of the desired double-stranded oligonucleotide by dideoxy sequence analysis using the commercially available forward and reverse sequencing primers. One clone found to contain the correct insert was designated pSSU 9/10.

Five micrograms of oligodeoxynucleotides SSU oligo 5 and SSU oligo 6 were phosphorylated as described above.

SSU oligo 5 5′-TGCGGAATGTGTGCGAACGTGGATGACTGAATGGA
TCCGGTAC -3′ (SEQ ID NO:15)

SSU oligo 6 5′-CGGATCCATTCAGTCATCCACGTTCGCACACATTC -3′
(SEQ ID NO:16)

Two μL aliquots of each phosphorylated primer were then combined, diluted to 40 μL with H_2O, heated to 70°C for 20 min and allowed to cool to ambient temperature.

The bacterial plasmid SSU.NP1 was digested to completion with Kpn I and BspMI and dephosphorylated with calf intestinal alkaline phosphatase. One mg of this DNA was then ligated with 1 μL of the dilute mixture of phosphorylated SSU oligo 5 and SSU oligo 6 as described above. The ligation reaction was diluted to 50 μL with H_2O and used to transform competent Max Efficiency *E. coli* HB101 as described above. Plasmids from individual antibiotic resistant colonies were analyzed for incorporation of the desired double-stranded oligonucleotide by digestion with Bam HI. One clone found to contain the correct 1.33 kbp Bam HI insert was designated pNP1 5/6.

The plasmid pNP1 5/6 was digested to completion with Kas I and Bam HI and the digestion products were separated by electrophoresis using a 6.5% agarose gel. The 1.3 kbp Kas I/Bam HI fragment was recovered from the gel. 400 ng of this DNA was ligated with 1 μg of the plasmid pSSU 9/10 that had been digested to completion with Kas I and Bam HI and dephosphorylated as described above. The ligation reaction was diluted five fold with H_2O and used to transform competent Max Efficiency *E. coli*

HB101 as described above. Plasmids from individual antibiotic resistant colonies were analyzed by digestion with Bbs I and Xho I until a clone found was found that contained the desired 1.3 kbp insert. This plasmid was designated pSSUBbs/Bam.

The plasmid pSSU Bbs/Bam was digested to completion with Bbs I and Xho I and the digestion products were separated by electrophoresis using a 6.5% agarose gel and the 1.35 kbp Bbs I/Xho I fragment was recovered from the gel. Three hundred ng of this DNA was ligated with 1 μg of the plasmid pGEX-2TM that had been digested to completion with BamH I and Xho I and dephosphorylated as described above. The ligation reaction was diluted five fold with H_2O and used to transform competent Max Efficiency *E. coli* DH5α as described above. Plasmids from individual antibiotic resistant colonies were analyzed by restriction endonuclease digestion until a clone found was found that contained the desired ALS small subunit coding region. This plasmid was designated pGEX-SSU.

EXAMPLE 3

Expression of recombinant plant acetolactate synthase small subunit

Expression of Trx-HIS/SSU

The strain GI724 harboring the plasmid pTrx-HIS/SSU (designated GI724/Trx-HIS/SSU) was struck onto a fresh RMG plate containing 100 μg/mL ampicillin and grown overnight at 30°C. The next day, a single colony was then used to inoculate a 20 mL culture of RM (6 g/L Na_2HPO_4, 3 g/L K_2HPO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 20 g/L amicase (acid casein hydrolysate; Sigma Chemical Co., St. Louis, MO), 1 mM $MgCl_2$, pH 7.4, 1% glycerol) supplemented with ampicillin to 100 μg/mL was grown overnight at 30°C. On the third day, the A_{550} of the culture was measured and 1 L of induction media [6 g/L Na_2HPO_4, 3 g/L K_2HPO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 0.2% amicase (acid casein hydrolysate; Sigma Chemical Co., St. Louis, MO), 1 mM $MgCl_2$, pH 7.4, 0.5% glucose, 100 μg/mL ampicillin] was inoculated with a sufficient volume of the overnight culture to give an initial A_{550} of 0.1. This fresh culture was grown to an A_{550} of 0.6 and 10 mL of a 10 mg/mL solution of tryptophan was added. The culture was incubated at 28°C for 3-4 h and then chilled to 0°C in a ice bath and subjected to centrifugation at 8000X g for 10 min. The supernatant was discarded and the cell pellet was frozen at -80°C.

Expression of GST-SSU

A single colony of BL21 transformed with pGEX-SSU from an LB/Amp (100 μg/mL) plate was used to inoculate 10 mL of LB containing 100 μg/mL ampicillin. These cultures were allowed to incubate overnight with shaking at 28°C. Two milliliters of the overnight culture was used inoculate each liter of LB containing 100 μg/mL ampicillin. The cultures were grown at 28°C until the absorbency at 600 nm was equal to 0.6. At this stage, 1 mL of 0.1 g/mL IPTG was added to each culture. The

cultures were allowed to remain at 28°C for 5 h with shaking after which time the cultures were placed on ice and the cells were harvested by centrifugation at 8000 rpm in a GS3 rotor for 15 min. The cells were stored frozen at -80°C until use.

EXAMPLE 4

Purification of plant ALS small subunit

Purification of Trx-HIS/SSU

The small subunit of plant ALS can be purified, using the appropriate affinity resin, as a fusion protein with a variety of affinity tags including but not exclusive to thioredoxin, hexahistidine and glutathione-S-transferase. The following example is for purification of the small subunit as a fusion protein with thioredoxin. Approximately 11 g of GI724 cells harboring the plasmid pTrx-HIS/SSU were suspended in 33 mL of buffer containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9 and 0.5% Triton X-100. The resuspended cells were sonicated using a half-inch horn on a Heat Systems Sonicator. Cells were sonicated, on ice, at full power using a 50% pulsed duty cycle for 15 s followed by 45 s rest. This sequence was repeated 19 times. The cell lysate was centrifuged at 10,000 rpm for 15 min in an SS34 rotor. The supernatant was transferred to a clean centrifuge tube and the centrifugation was repeated until the supernatant was clear. The lysate supernatant was added to 20 mL of ThioBond™ resin (Invitrogen). Binding of the fusion protein was allowed to proceed for 45 min by gently rocking the resin at room temperature. The resin was allowed to settle and the liquid was decanted. Unbound protein was removed by washing the resin with 40 mL of buffer containing 50 mM MOPS, pH 7.0 and 1 mM EDTA. The resin was equilibrated with this buffer by gentle rocking and then allowed to settle and the supernatant was removed. This procedure was repeated twice. The fusion protein was eluted by washing the resin with buffer containing 50 mM MOPS pH 7.0, 1 mM EDTA, 10 mM DTT and 10% glycerol. After equilibration, the supernatant was decanted and the procedure was repeated. The fusion protein was eluted with a total of 100 mL of buffer. The eluted protein was concentrated using an Amicon concentrator and a PM30 membrane. The sample was dialyzed into PBS buffer containing 10% glycerol and then frozen at -80°C for storage.

Purification of GST-SSU

Approximately 50 g of BL21 cells harboring the plasmid pGEX-SSU were suspended in 100 mL of 1 X PBS buffer. The cell suspension was subjected to three passes through a microfluidizer. A 10% Triton X-100 solution and a 20% sarkosyl solution were each added to the cell lysate to a final concentration of 1%. The solution was allowed to equilibrate for 30 min at room temperature with gentle agitation. The cell debris was then centrifuged at 10,000 rpm in a GSA rotor for 30 min. The supernatant was removed and added to 100 mL of glutathione agarose (Sigma). The

resin and lysate were allowed to equilibrate at room temperature for 30 minutes with gentle rocking. The supernatant was removed by centrifugation at 500 x g (swinging bucket rotor) for 5 minutes. The supernatant was decanted and the resin was washed with 200 mL of 1 X PBS. The resin and PBS were allowed to equilibrate at room temperature for 10 minutes and with rocking and the supernatant was removed by centrifugation as above. The washing procedure was repeated a total of 4 times. The bound GST-SSU was eluted by adding 200 mL of 25 mM Ches pH 9 containing 10 mM reduced glutathione. The resin was equilibrated with the Ches buffer for 10 min with gentle rocking at room temperature. The supernatant was collected by centrifugation as above. The elution procedure was repeated a total of three times. The eluted protein was concentrated to 9 mL using a Millipore ultrafiltration apparatus. The protein was dialyzed extensively to remove the glutathione and then stored frozen at -80°C.

EXAMPLE 5

Expression and Purification of an *Arabidopsis thaliana* ALS large subunit

The ALS large subunit may also be cloned into a variety of expression vectors and purified as a fusion protein using numerous affinity chromatography strategies specific for, but not exclusive to, thioredoxin, glutathione-S-transferase and hexahistidine. The procedure described below can be used for any ALS large subunit cloned into a pGEX expression vector ["GST Gene Fusion System" Pharmacia Biotech, 1996; Bernasconi et al., *J. of Biol. Chem. 270*: 17381-17385 (1995)].

The plasmid pGATX was created by cloning the 5.8 kbp Xba I fragment of the genomic lambda phage clone 7 [Mazur et al. *Plant Physiol. 85*:1110-111 (1987)], into the plasmid vector pGEM1 (Promega). This 5.8 kbp Xba I fragment contains a complete copy of the *Arabidopsis thaliana* ALS large subunit gene. The plasmids pGEX-2TM (see Example 2) and pGATX were both digested to completion with Nco I and the digests then placed on Qiagen QIAquick™ columns and the Nucleotide Removal Kit protocol followed. The final column eluant was adjusted to 1X React 4 (Gibco BRL) and the DNAs were digested to completion with PinAI. The pGEX-2TM digest was heated to 65° C for 10 minutes to destroy remaining PinAI activity and the DNA was dephosphorylated by incubating it at 37°C for 30 min. with 1 unit of calf intestinal alkaline phosphatase. Both pGEX-2TM and pGATX were subjected to electrophoresis using a 1% agarose gel. The 2 kbp fragment from pGATX and the 4.9 kbp fragment from pGEX-2TM were removed from the gel and the DNAs were recovered using a QIAquick™ Gel Extraction Kit according to the manufacturer's instructions. Purified DNAs were precipitated, re-dissolved and quantified as above. Approximately 90 ng of 2.0 kbp pGATX fragment were mixed with 120 ng of the NcoI/PinAI digested pGEX-2TM and mixture was heated for 5 minutes at 45° C followed by cooling on ice. T4 DNA ligase buffer and 1 unit of T4 DNA ligase were

mixed with the fragments and mixture was incubated for 4 hours at ambient temperature. An aliquot of the ligation mixture was used to transform competent *E. coli* DH5α as described above and aliquots of the transformation mixture were spread onto LB plates containing 100 μg/mL carbenicillin. The plates were incubated overnight at 37° C and plasmids were isolated from carbenicillin-resistant bacterial colonies were analyzed for the presence of the ALS insert by double digestion with PinAI and NcoI. One plasmid thus isolated was designated pGEX-OCM2 was then transformed into *E. coli*. BL21(DE3) using methods known to those skilled in the art.

A single colony of BL21(DE3) transformed with pGEX-OCM2 (encoding a fusion protein designated GST-ALS, comprising the glutathione-S-transferase fused to the *Arabidopsis thaliana* large subunit of acetolactate synthase, including the chloroplast transit peptide) from an LB/Amp (100 μg/mL) plate is used to inoculate 10 mL of LB containing 100 μg/mL carbenicillin. These cultures were allowed to incubate overnight with shaking at 30°C. Two milliliters of the overnight culture was used inoculate each liter of LB containing 100 μg/mL carbenicillin. The cultures were grown at 30°C until the absorbency at 600 nm was equal to 0.6. At this stage, 1 mL of 0.1 g/mL IPTG was added to each culture. The cultures were allowed to remain at 30°C for 4 h with shaking after which time the cultures were placed on ice and the cells were harvested by centrifugation at 8000 rpm in a GS3 rotor for 15 min. The cells were stored frozen at -80°C until use.

Approximately 11 grams of cells were resuspended in 20 mL of PBS buffer containing Complete™ protease inhibitors (Boehringer Mannheim, Indianapolis, IN). The cells were lysed by sonication (Heat Systems) 19x15 s with 45 s rest on ice using a microtip at power level 4 and 50% duty cycle. The appropriate amount of a 20% Triton X-100 solution was added so that the final concentration of Triton in the lysate was 1%. The lysate was gently rocked at 4°C for 30 min. The cell debris was removed by centrifugation at 12,000 rpm for 15 min in a SS34 rotor. The supernatant was removed and the cell pellet was discarded. Half of the cell lysate supernatant was added to a 2 mL column of glutathione Sepharose 4B (Pharmacia Biotech) equilibrated with PBS. The eluant was collected for all washes and elutions. The column was washed with 20 mL of PBS. The bound fusion protein was eluted using 5 mM glutathione (reduced) in PBS at pH 8.0. One column volume of elution buffer was added to the column and allowed to run into the column. The bottom of the column was capped and the column was allowed to sit at room temperature for 10 min. The column effluent was collected as another 2.66 mL of elution buffer was added. The process was repeated 3 times. The elution fractions were combined and dialyzed against 50 mM Hepes, pH 7.0 containing 10% glycerol and 0.1 mM FAD for several hours and then concentrated to 9 mL using a Centriprep 10 (Amicon).

EXAMPLE 6

Expression and purification of *Nicotiana plumbaginifolia* ALS large subunit

Equimolar amounts of oligodeoxynucleotides mt704+ (SEQ ID NO:17) and mt800- (SEQ ID NO:18) were combined in 100 uL of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, heated briefly in a boiling water bath and slow cooled to 50°C. The resulting double stranded DNA fragment was digested to completion with Nde I and Eco0109I.

The plasmid ALS10 that contains a full length cDNA copy of the *Nicotiana plumbaginifolia* ALS gene in the plasmid vector pBluescript was used as the source of the ALS coding region for pMTDRALS. The sequence of this cDNA is shown in SEQ ID NO:19. ALS10 was digested to completion with Xho I and Eco0109I, and the digestion products were separated by agarose gel electrophoresis. The 1.9 kbp Xho I/ Eco0109I ALS10 DNA fragment was excised from the gel and the DNA was recovered using a QIAquick™ Gel Extraction Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Equimolar amounts of Nde I/Eco0901I digested double-stranded oligonucleotide mt704+ /mt800- DNA, and 1.9 kbp Xho I/ Eco0109I ALS10 DNA were ligated together overnight. DNA ligase was inactivated by heating the mixture to 65°C for 1 hr and the ligation products were digested to completion with Nde I and Xho I. The digestion products were separated by agarose gel electrophoresis and the 2.0 kbp Xho I/ Nde I fragment was excised from the gel and the DNA was recovered as described earlier. The plasmid pET24a (Novagen, Inc., Madison, WI) was digested to completion with Nde I and Xho I and the DNA was dephosphorylated with calf intestinal alkaline phosphatase. Equimolar amounts of Nde I/Xho I digested and dephosphorylated pET24a and the 2.0 kbp Xho I/ Nde I digestion product were ligated together and an aliquot of the ligation mixture was used to transform competent *E. coli* DH5α as described above. Aliquots of the transformation mixture were spread onto LB plates containing 100 μg/ml kanamycin. The plates were incubated overnight at 37° C and plasmids isolated from kanamycin-resistant bacterial colonies were analyzed for the presence of the insert encoding the ALS large subunit by double digestion with Nde I and Xho I. One such plasmid, designated pMTDRALS was then transformed into *E. coli*. BL21(DE3) using methods known to those skilled in the art. This plasmid directs the expression of the *Nicotiana plumbaginifolia* ALS large subunit.

A single colony of BL21 transformed with pMTDRALS grown on an LB/kanaymycin (30 μg/mL) plate was used to inoculate 50 mL of LB/kanamycin. This culture was grown overnight at 37°C with agitation. This overnight culture was in turn used to inoculate 1 liter of minimal M9 medium supplemented with 2 mL 1M $MgSO_4$, 1 ml $CaCl_2$, 50 μL 1% vitamin B1, 40 ml of 10% casamino acids and 30 μg/mL kanamycin. The cells were grown until OD_{600} was equal to 0.6 and expression was

induced by the addition of IPTG to a final concentration of 0.5 mM. The cells were harvested by centrifugation 5 hours later.

Cells were washed and then resuspended in two volumes of 20mM Tris, pH 7.8 containing 1mM EDTA, 1mM β-mercaptoethanol and 0.1mM PMSF. The suspension was subjected to three passes through a microfluidizer and the lysed cell debris removed by centrifugation at 18000 rpm on a SS34 rotor. The proteins in the supernatant were fractionated by ion exchange chromatography using a DE 52 column equilibrated with 50mM MOPS, pH 6.85, 1mM EDTA and 1mM β-ME. Protein was eluted with a linear NaCl gradient between 0 and 0.5 M. The tubes containing ALS activity were combined and the protein precipitated with 50% ammonium sulfate. The precipitated protein was centrifuged at 11000 rpm (GSA rotor) and the pellet resuspended in Tris, pH 7.2 containing 1mM EDTA, 1mM DTT and 20 μM FAD. The solutions were kept dark. The redissolved protein was desalted and fractionated using an S300 filtration column equilibrated with the FAD containing Tris buffer. The tubes containing ALS activity were combined, concentrated and the material loaded onto a MonoQ column equilibrated with Tris, pH 7.2 containing 1mM EDTA, 1mM DTT and developed with a linear NaCl gradient to 0.5M. The resulting enzyme was concentrated and washed with Tris buffer and stored as frozen aliquots in 10% glycerol. The exposure of solutions to light was minimized during this purification.

EXAMPLE 7

Effect of the small subunit on ALS-LSU activity

As discussed above, one way to prepare the ALS holoenzyme is to mix purified or partially purified large subunit with purified or partially purified small subunit. A typical subunit mixing experiment which demonstrates the effect of the small subunit on the specific activity of the LSU consists of the samples and the results shown in Table 1.

Table 1

Sample	GST-LSU[1] (1 mg/mL)	LSU[2] (0.7 mg/mL)	Trx-HIS/SSU[3] (2 mg/mL)	BSA (2 mg/mL)	Specific Activity[4] before thrombin addition	Specific Activity[4] after thrombin addition
1	20 μl	-	-	10 μl	0.3	0.04
2	20 μl	-	10 μl	-	0.9	1.1
3	-	5 μl	-	10 μl	1.1	0.40
4	-	5 μl	10 μl	-	5.5	6.5

[1] *A. thaliana* LSU expressed as a glutathione-S-transferase fusion protein from plasmid pGEX-OCM2.

[2] *N. plumbaginifolia* LSU from plasmid pMTDRALS.

[3] *N. plumbaginifolia* SSU expressed as a thioredoxin fusion from plasmid pTrx-HIS/SSU.

[4] Expressed as units/mg LSU.

Each sample contained 3 µl of 10X PBS and distilled, deionized water was added to each sample to bring the final volume to 30 µL. When small subunit was not added to the sample, an equivalent amount of BSA was added as a control protein. The Trx-HIS/SSU used in these experiments contains a thrombin cleavage site. Thus, the addition of thrombin leads to cleavage of the fusion to yield free SSU and Trx-HIS. Two different large subunits are used in this example. One ALS-LSU (GST-LSU) is a fusion between GST and the LSU of *Arabidopsis* ALS which contains the complete chloroplast transit peptide on the N-terminus of the LSU. Upon the addition of thrombin, the GST-LSU fusion is cleaved to give free GST and *Arabidopsis* LSU. The other ALS-LSU (LSU) is from *Nicotiana plumbaginifolia* and contains only a partial chloroplast transit peptide on the N-terminus as described above and contains no thrombin cleavage site. Each sample was assayed for ALS activity (see below) prior to thrombin addition. After the initial activity had been measured, 1 µL of thrombin (Pharmacia BioTech, 1 unit/µL) was added to each sample and the samples were incubated at room temperature for 2 h. Thrombin cleavage was shown to be complete by SDS Page analysis and no proteolytic degradation of the *Nicotiana* LSU was observed. Each sample was again assayed for activity. The assay results are shown in the last two columns of Table 1 where specific activities are calculated based upon large subunit concentration and are reported in terms of units/mg LSU.

The results in Table 1 show that the presence of the small subunit increases the specific activity of the LSU before and after thrombin cleavage of the fusion protein regardless of whether the LSU is from *Arabidopsis* or *Nicotiana* and that the SSU positively affects the stability of the LSU activity. A comparison of the specific activity of sample 1 and 2 before thrombin addition shows that when Trx-HIS/SSU is added to GST-LSU from *Arabidopsis* there is a 3-fold increase in specific activity even with fusion proteins attached to both large and small subunits. A comparison of the specific activities of samples 3 and 4 prior to thrombin addition shows that the presence of the Trx-HIS/SSU increases the specific activity of the *Nicotiana* LSU by a factor of 5. After thrombin addition to sample 1, the specific activity of the *Arabidopsis* LSU, now minus the GST fusion, decreases from 0.3 to 0.04 units/mg LSU. However, in the presence of SSU and thrombin, the specific activity of the *Arabidopsis* LSU increases slightly compared to the same sample in the absence of thrombin (sample 2) to 1.1 units/mg LSU representing more than a 20 fold increase in specific activity compared to the *Arabidopsis* LSU alone. In sample 3, the addition of thrombin does not cleave the *Nicotiana* large subunit, but the enzyme loses some activity during the incubation period. Control samples identical to 3 but which do not contain thrombin showed a similar decrease in specific activity over the 2 hour incubation period (data not shown). However, in the presence of the SSU and thrombin, the specific activity of the

Nicotiana LSU increases from 5.5 to 6.5 (sample 4). Considering the loss in specific activity observed for the *Nicotiana* LSU alone during the incubation time, the SSU serves to not only increase the specific activity of the LSU but also serves to stabilize the LSU. Samples identical to 1 and 3 but which do not contain BSA showed similar specific activities to those with BSA. Thus, the effect of the small subunit cannot be mimicked by interactions with a non-specific protein. These results show not only that the small subunit of plant ALS increases the specific activity of the large subunit but also that the SSU from *N. plumbaginifolia* can affect the activity of an *Arabidopsis* large subunit as well. Thus the small subunit from one species of plant may affect the activity of a large subunit from another species.

ALS assays were conducted using the following reaction mixture containing 100 mM sodium phosphate pH 7.6, 0.5 mM dithiothreitol, 1 mM $MgCl_2$, 100 μM thiamine pyrophosphate, and 100 μM flavin adenine dinucleotide. The assay was be conducted in a microtiter plate wherein each well contained 80 μL of assay mix and 10 μL of 500 mM sodium pyruvate. The mixture was allowed to equilibrate for 5 min at 37°C. The desired amount of enzyme to be assayed was then added to the well and mixed by gentle shaking. The plate was incubated for the desired reaction time at 37°C. The reaction was quenched by the addition of 10 μL 3 M H_2SO_4 to each well. The contents of the plate were mixed well with gentle shaking and incubated at 60°C for 15 min. The amount of acetoin produced was detected by the rapid addition of 100 μL of 0.5% creatine and 100 μL of 5% α-naphthol in 2.5 M NaOH. The contents of the plate were mixed and the plate was incubated for 15 min at 60°C uncovered. The plate was cooled to room temperature for 5 min with constant gentle shaking and the absorbency at 530 nm was read. The specific activity can be calculated based upon the concentration of the large subunit given that 1 μmole of acetoin produces an absorbency of 0.35 under these conditions.

EXAMPLE 8

A holoenzyme mixture was prepared containing 50 μL of *N. plumbaginifolia* LSU (0.7 mg/mL; encoded by pMTDRALS), 60 μL GST-SSU (1.22 mg/mL; encoded by pGEX-SSU)), 10 μL distilled, deionized water, and 180 μL of a buffer/cofactor mixture containing 100 mM phosphate, pH 7.6, 10% glycerol, 0.5 mM DTT, 2 mM $MgCl_2$, 200 μM FAD and 200 μM TPP. The fusion proteins were cleaved by the addition of 1 μL of Thrombin (0.8 units/mL; Novagen) to the mixture and the solution was allowed to incubate at 25°C for several hours. A microtiter plate was prepared in which each well contains 80 μL of assay mix (see ALS assay procedure above) and 10 μL of a stock solution containing the desired concentration of the inhibitor to be tested. The plate was incubated at 37°C for 5 min and then 1 μL of the holoenzyme mixture was added to each well. The reaction was started by the immediate addition of 10 μL of 500 mM

sodium pyruvate to each well. The contents of the plate were mixed well by brief agitation and then the plate was incubated at 37°C for 30 min prior to quenching with 3 M H_2SO_4. The acetoin produced was detected as described above under ALS assays. The results of such a study using the commercial herbicides imazequin (Scepter®) and imezethapyr (Pursuit®) are shown in Table 2.

Table 2

Compound	I_{50} (μM)
imazequin	0.80
imezethapyr	4.0

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) ADDRESSEE: E. I. DUPONT DE NEMOURS AND COMPANY
(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: UNITED STATES OF AMERICA
(F) ZIP: 19898
(G) TELEPHONE: 302-992-4926
(H) TELEFAX: 302-773-0164
(I) TELEX: 6717325

(ii) TITLE OF INVENTION: USE OF THE SMALL SUBUNIT OF PLANT ACETOLACTATE SYNTHASE FOR NEW HERBICIDE DISCOVERY

(iii) NUMBER OF SEQUENCES: 19

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE, 3.50 INCH
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: MICROSOFT WORD FOR WINDOWS '95
(D) SOFTWARE: MICROSOFT WORD 7.0

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/039,148
(B) FILING DATE: FEBRUARY 24, 1997

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: MAJARIAN, WILLIAM R.
(B) REGISTRATION NUMBER: 41,173
(C) REFERENCE/DOCKET NUMBER: BA-9132

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 611 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGCATTCGGC ACGAGCGAAA ATCCACTTAC ATCTGTAAAT AGGACAGTTA ATGGCAGTTT 60

NGGTCAACCA TCCAATGCTG GGGGTGATGT TTATCCTGTG GAATCTTACG AGAGCTTATC 120

AGTGAACCAT GTACTTGATG CTCATTGGGG TGTTCTGGAT GATGATGATG CGACTGGACT 180

TCGCTCGCAT ACTCTCTCCA TCCTTGTGAA TGACTGTCCT GGTGTCCTCA ACATTGTAAC 240

AGGAGTCTTT GCTCGCAGGG GCTACAATAT ACAGAGCCTT GCTGTTGGCC CAGCTGAGAA 300

GGAAGGCATT TCGCGGATTA CAACAGTNGT NCCTGGTACT GTTGAATCCA TTGAGANAGT 360

NAGNTCAGCC AGCTTTACAA GCTTATTGAT GTGTAACGAA AGTTCCATGA CATTACCCAC 420

TCACCTTTTG CTGAAAGGGA ACTTATCCTT ATCTAAGGTT TCTGTCAACA CTGCTGCTCG 480

GAAGGAAATC CTACATATTG CTCAAATCTT CCGAGCAAAA CCTGTTGATG TTTCTGACCA 540

CACAGTAACG CTTCAGCTTA CTGGAGATCT TGACAAGATG GTTGACACTA CAAAGGTTAT 600

TGGAGCCATA T 611

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCAGTACGA GCAATTTCTC 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTGGCTCCTT GGATAGATCT 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1861 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 181..1527

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGATTCGGCA CGAGGGCGGC TGTTTCAACT CCCTTCAACA GCTCTCTAAA TAAACCCTTC 60

CTTTCTCTCT ATTCCTCTAT TCCCCATTTG GGTTTCTCCC AAGTTGTTCA ATTTCCAACT 120

TTGAAACCAA AATTTCTTCT TTCAAAGATC AAAACAGTTG TAATTTCTGC GCCAAAGCTG 180

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ATG GAA CAT ATA CAA ACG AGG ACG ACT CTC TCA CAA CTT TCG ACT CTT    228
Met Glu His Ile Gln Thr Arg Thr Thr Leu Ser Gln Leu Ser Thr Leu
  1               5                  10                  15

CCA TCA GAC AAA AGA CTA GGC GCC ATA CGA TTC AAG TGT TTG TTG GTG    276
Pro Ser Asp Lys Arg Leu Gly Ala Ile Arg Phe Lys Cys Leu Leu Val
             20                  25                  30

ATG AAA GTG GAA ATG ATC AAT CGT ATT GCG GGA GTC TTT GCT CGA AGA    324
Met Lys Val Glu Met Ile Asn Arg Ile Ala Gly Val Phe Ala Arg Arg
         35                  40                  45

GGG TAC AAT ATT GAG TCT CTT GCT GTT GGT CTA AAC AAG GAC AAG GCT    372
Gly Tyr Asn Ile Glu Ser Leu Ala Val Gly Leu Asn Lys Asp Lys Ala
     50                  55                  60

CTT TTT ACT ATA GTT GTT TCT GGA ACT GAA AGG GTG TTA CAG CAA GTT    420
Leu Phe Thr Ile Val Val Ser Gly Thr Glu Arg Val Leu Gln Gln Val
 65                  70                  75                  80

ATG GAA CAG TTA CAA AAG CTT GTA AAT GTG ATC AAG GTT GAA GAT CTA    468
Met Glu Gln Leu Gln Lys Leu Val Asn Val Ile Lys Val Glu Asp Leu
                 85                  90                  95

TCC AAG GAG CCA CAA GTT GAA CGA GAA TTA ATG CTT ATT AAA ATC AGC    516
Ser Lys Glu Pro Gln Val Glu Arg Glu Leu Met Leu Ile Lys Ile Ser
            100                 105                 110

GCC GAT CCA AAA TAC CGC GCA GAG GTT ATG TGG TTG GTG GAC GTT TTC    564
Ala Asp Pro Lys Tyr Arg Ala Glu Val Met Trp Leu Val Asp Val Phe
        115                 120                 125

AGG GCA AAA ATT GTG GAT ATA TCT GAT CAA TCT CTG ACT ATT GAG GTA    612
Arg Ala Lys Ile Val Asp Ile Ser Asp Gln Ser Leu Thr Ile Glu Val
    130                 135                 140
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ACT GGA GAT CCA GGG AAG ATG GTG GCT GTT CAG AGG AAC TTA AGT AAA     660
Thr Gly Asp Pro Gly Lys Met Val Ala Val Gln Arg Asn Leu Ser Lys
145                 150                 155                 160

TTT GGA ATT AGA GAA ATT GCT CGT ACT GGG AAG ATT GCC TTG AGA AGA     708
Phe Gly Ile Arg Glu Ile Ala Arg Thr Gly Lys Ile Ala Leu Arg Arg
            165                 170                 175

GAA AAA ATG GGG GAA TCT GCT CCT TTT TGG CGG TTT TCA GCA GCA TCA     756
Glu Lys Met Gly Glu Ser Ala Pro Phe Trp Arg Phe Ser Ala Ala Ser
        180                 185                 190

TAC CCA GAT CTT GAA GGT GCA ATG TCT GCT GGT ACT ATT TCG AGG ACA     804
Tyr Pro Asp Leu Glu Gly Ala Met Ser Ala Gly Thr Ile Ser Arg Thr
    195                 200                 205

ATC AAG AGG ACC CCT AAT GGA GAA TCT ATG TCT ATG GCT GAG GGA GAT     852
Ile Lys Arg Thr Pro Asn Gly Glu Ser Met Ser Met Ala Glu Gly Asp
210                 215                 220

GTC TAT CCT GTT GAG ACA GAT GAC AAC TCT GGA GTC AGT CAA GTT CTT     900
Val Tyr Pro Val Glu Thr Asp Asp Asn Ser Gly Val Ser Gln Val Leu
225                 230                 235                 240

GAT GCT CAC TGG GGT GTT CTC AAT GAT GAA GAT ACA AGT GGG CTT CGC     948
Asp Ala His Trp Gly Val Leu Asn Asp Glu Asp Thr Ser Gly Leu Arg
            245                 250                 255

TCA CAT ACT TTG TCA ATG CTT GTG AAT GAC ACT CCT GGA GTT CTC AAC     996
Ser His Thr Leu Ser Met Leu Val Asn Asp Thr Pro Gly Val Leu Asn
        260                 265                 270

ATA GTC ACC GGA GTT TTT GCT CGA CGA GGG TAT AAC ATC CAA AGT TTA    1044
Ile Val Thr Gly Val Phe Ala Arg Arg Gly Tyr Asn Ile Gln Ser Leu
    275                 280                 285

GCT GTT GGA CAT GCT GAA GTT GAG GGG CTT TCT CGT ATT ACA ACG GTT    1092
Ala Val Gly His Ala Glu Val Glu Gly Leu Ser Arg Ile Thr Thr Val
290                 295                 300

GTT CCT GGC ACA GAT GAG TCA GTT AGC AAG TTG GTG CAG CAA CTA TAT    1140
Val Pro Gly Thr Asp Glu Ser Val Ser Lys Leu Val Gln Gln Leu Tyr
305                 310                 315                 320

AAG TTG GTT GAT ATT CAT GAG GTT CGG GAT ATT ACT CAC CTC CCA TTT    1188
Lys Leu Val Asp Ile His Glu Val Arg Asp Ile Thr His Leu Pro Phe
            325                 330                 335

GCG GAA AGA GAA CTA ATG TTG ATA AAG ATT GCT GTG AAT GCT GCA GCG    1236
Ala Glu Arg Glu Leu Met Leu Ile Lys Ile Ala Val Asn Ala Ala Ala
        340                 345                 350

CGC CGC AAT GTT CTT GAC ATT GCC AGC ATT TTC AGA GCA AAA GCT GTT    1284
Arg Arg Asn Val Leu Asp Ile Ala Ser Ile Phe Arg Ala Lys Ala Val
    355                 360                 365

GAT GTG TCT GAC CAC ACT ATA ACT CTT GAG CTT ACA GGA GAT TTG CAT    1332
Asp Val Ser Asp His Thr Ile Thr Leu Glu Leu Thr Gly Asp Leu His
370                 375                 380

AAG ATG GTT CGT TTG CAG CGG CTA CTA GAG CCT TAT GGT ATT TGT GAG    1380
Lys Met Val Arg Leu Gln Arg Leu Leu Glu Pro Tyr Gly Ile Cys Glu
385                 390                 395                 400
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GTA GCG CGA ACA GAC GTC TGG CAC TGG TAC GTG AAT CAG GTG TGG ATT 1428
Val Ala Arg Thr Asp Val Trp His Trp Tyr Val Asn Gln Val Trp Ile
405 410 415

CGA AGT ACT TGC GAG GAT ATT CAT ACC CTT TGT AGT CTA AAG TCC TCG 1476
Arg Ser Thr Cys Glu Asp Ile His Thr Leu Cys Ser Leu Lys Ser Ser
420 425 430

AAC CTA AGG AAA ATA CCT GCT CTT TGC GGA ATG TGT GCG AAC GTG GAT 1524
Asn Leu Arg Lys Ile Pro Ala Leu Cys Gly Met Cys Ala Asn Val Asp
435 440 445

GAC TGAATGATAC GGAGATGGTC TCGTGCCCCA GCTGTGACCC TACCGCTTTC 1577
Asp

AAGTCAAGTT TGCATGCTTT TAGCTTGAGG TAGTTGCAAG TTTATGAAAA TGAATAGGGT 1637

ACAATTTGAC CATTCTATGA ACAAAAGCGG ACCCAGTCTT TAAGATACAA GAGTTTTCAG 1697

CTCTTTTCTT TTCACCTTTA TTCTTTAGCA GAGATAGTTC TTCAATAAGC TTCACTGAAA 1757

ATGATTGACG TCGCGTCGTC ATTTGACTTA CAAGTAAAAT ATGACTAGCA TTTTTGGTTT 1817

TTAAATCTGA AAGTGATATG AAACATAAAA AAAAAAAAAA AAAA 1861

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTATACGAGG ATCCTCTAGA G 21

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCGACTCTAG AGAGTCCTCG TATAC 25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGAATTCTCC ATATGCACCA TCATCATCAT CATAGCGATA AAATTATTCA C 51

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCTGTACGAT TACTGCAGGT C 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AACAACAACG ATATCAGACA AAAGACTAGG CGCCA 35

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AACAACGGAT CCAACCAACT TATATAGTTG CTGCACCA 38

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GATCCATGGT CGACTCGAGA CCGGTG 26

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AATTCACCGG TCTCGAGTCG ACCATG 26

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATCTGAAGA CAACATGATC AGACAAAAGA CTAGGCGCCA ACAACAAGGA TCC 53

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCGAGGATCC TTGTTGTTGG CGCCTAGTCT TTTGTCTGAT CATGTTGTCT TCA 53

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TGCGGAATGT GTGCGAACGT GGATGACTGA ATGGATCCGG TAC 43

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGGATCCATT CAGTCATCCA CGTTCGCACA CATTC 35

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 114 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTCATTTCCA CTCATATGAA AGTTTCCGAG ACCCAAAAAA CCGAAACTTT CGTTTCTAGA 60

TTTGCCCCGG ACGAACCCAG AAAGGGTTCC GACGTTCTCG TGGAGGCCCT CGAA 114

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 114 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTCGAGGGCC TCCACGAGAA CGTCGGAACC CTTTCTGGGT TCGTCCGGGG CAAATCTAGA 60

AACGAAAGTT TCGGTTTTTT GGGTCTCGGA AACTTTCATA TGAGTGGAAA TGAC 114

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2702 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 492..2492

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

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GAATTCACCT TCGGAGGGAA CCAGCTACTA GACGGTTCGA TTAGTCTTTC GCCCCTATAC   60

CCAAGTCAGA CGAACGATTT GCACGTCAGT ATCGCCTGCG GCCTCCACCA GAGTTTCCTC  120

TCTCTGCCCC GCTCAGGCAT AGTTCACCAT CTTTCGGGTC CCGACAGGTA TGCTCACACT  180

CGAACCCTTC ACAGAAGATC AAGGTCGGTC GGCGGTGCAC CTCAGGGGGA TCCCACCAAT  240

CAGCTTCCTT ACGCTTACGG GTTTACTCGC CCGTTGACTC GCACACATGC GGAATTCCGG  300

TCAGGTTAAG GTAATTGCTA AATTGGTTCA TCCAAATCTT TAAACCGTTC GACGCCGCTT  360

TCTCCAATCC GCCGCACAGC CAGTGTTGTC CATCTTCTAA CACCGCCTTG TACATTATAC  420

TCGAACCACT AGCTCCCAAA ATATAAGCAG ATGCTTTAAG CAATGTCTCT CCCAGCTCTC  480

ATTCAACAAT A ATG GCG GCG GCG GCT GCT CCA TCT CCC TCT TCT TCC GCT     530
            Met Ala Ala Ala Ala Ala Pro Ser Pro Ser Ser Ser Ala
              1               5                  10

TTC TCC AAA GCC CTA ATG TCC TCC TCC TCC AAA TCC TCC ACC CTC CTC     578
Phe Ser Lys Ala Leu Met Ser Ser Ser Ser Lys Ser Ser Thr Leu Leu
     15                  20                  25

CCT AGA TCC ACT TTT CCT TTC CCC CAC CAC CCC CAC AAA ACC ACC CCA     626
Pro Arg Ser Thr Phe Pro Phe Pro His His Pro His Lys Thr Thr Pro
 30                  35                  40                  45

CCA CCC CTC CAC CTC ACC CCC ACC CAC ATT CAC AGC CAA CGC CGT CGT     674
Pro Pro Leu His Leu Thr Pro Thr His Ile His Ser Gln Arg Arg Arg
                 50                  55                  60

TTC ACC ATC TCC AAT GTC ATT TCC ACT ACC CAA AAA GTT TCC GAG ACC     722
Phe Thr Ile Ser Asn Val Ile Ser Thr Thr Gln Lys Val Ser Glu Thr
            65                  70                  75

CAA AAA ACC GAA ACT TTC GTT TCC CGT TTT GCC CCG GAC GAA CCC AGA     770
Gln Lys Thr Glu Thr Phe Val Ser Arg Phe Ala Pro Asp Glu Pro Arg
        80                  85                  90

AAG GGT TCC GAC GTT CTC GTG GAG GCC CTC GAA AGA GAA GGG GTT ACG     818
Lys Gly Ser Asp Val Leu Val Glu Ala Leu Glu Arg Glu Gly Val Thr
     95                  100                 105

GAC GTT TTT GCG TAC CCA GGC GGC GCT TCC ATG GAG ATT CAC CAA GCT     866
Asp Val Phe Ala Tyr Pro Gly Gly Ala Ser Met Glu Ile His Gln Ala
110                 115                 120                 125

TTG ACT CGT TCA AGC ATC ATC CGC AAC GTG CTG CCG CGT CAC GAG CAG     914
Leu Thr Arg Ser Ser Ile Ile Arg Asn Val Leu Pro Arg His Glu Gln
                130                 135                 140

GGT GGT GTC TTC GCC GCT GAG GGT TAC GCA CGC GCC ACC GGC TTC CCC     962
Gly Gly Val Phe Ala Ala Glu Gly Tyr Ala Arg Ala Thr Gly Phe Pro
            145                 150                 155
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GGC GTT TGC ATT GCT ACC TCC GGC CCC GGT GCC ACC AAT CTC GTC AGT   1010
Gly Val Cys Ile Ala Thr Ser Gly Pro Gly Ala Thr Asn Leu Val Ser
        160                 165                 170

GGC CTC GCG GAC GCC CTA CTG GAT AGC GTC CCC ATT GTT GCT ATA ACC   1058
Gly Leu Ala Asp Ala Leu Leu Asp Ser Val Pro Ile Val Ala Ile Thr
    175                 180                 185

GGT CAA GTG CCA CGT AGG ATG ATC GGT ACT GAT GCT TTT CAG GAA ACT   1106
Gly Gln Val Pro Arg Arg Met Ile Gly Thr Asp Ala Phe Gln Glu Thr
190                 195                 200                 205

CCG ATT GTT GAG GTA ACT AGA TCG ATT ACC AAG CAT AAT TAT CTC GTT   1154
Pro Ile Val Glu Val Thr Arg Ser Ile Thr Lys His Asn Tyr Leu Val
                210                 215                 220

ATG GAC GTA GAG GAT ATT CCT AGG GTT GTA CGT GAG GCT TTT TTC CTT   1202
Met Asp Val Glu Asp Ile Pro Arg Val Val Arg Glu Ala Phe Phe Leu
            225                 230                 235

GCG AGA TCG GGC CGG CCT GGC CCT GTT TTG ATT GAT GTA CCT AAG GAT   1250
Ala Arg Ser Gly Arg Pro Gly Pro Val Leu Ile Asp Val Pro Lys Asp
        240                 245                 250

ATT CAG CAA CAA TTG GTG ATA CCC AAC TGG GAT CAG CCA ATG AGA TTG   1298
Ile Gln Gln Gln Leu Val Ile Pro Asn Trp Asp Gln Pro Met Arg Leu
    255                 260                 265

CCC GGT TAC ATG TCT AGG TTG CCG AAA TTG CCC AAT GAG ATG CTT TTA   1346
Pro Gly Tyr Met Ser Arg Leu Pro Lys Leu Pro Asn Glu Met Leu Leu
270                 275                 280                 285

GAA CAA ATT GTT AGG CTT ATT TCT GAG TCA AAG AAA CCT GTT TTG TAT   1394
Glu Gln Ile Val Arg Leu Ile Ser Glu Ser Lys Lys Pro Val Leu Tyr
                290                 295                 300

GTG GGG GGT GGG TGT TCG CAA TCG AGT GAG GAG TTG AGA CGA TTC GTG   1442
Val Gly Gly Gly Cys Ser Gln Ser Ser Glu Glu Leu Arg Arg Phe Val
            305                 310                 315

GAG CTC ACG GGT ATC CCC GTG GCA AGT ACT TTG ATG GGT CTT GGA GCA   1490
Glu Leu Thr Gly Ile Pro Val Ala Ser Thr Leu Met Gly Leu Gly Ala
        320                 325                 330

TTT CCA ACT GGG GAT GAG CTT TCC CTT TCA ATG TTG GGT ATG CAT GGT   1538
Phe Pro Thr Gly Asp Glu Leu Ser Leu Ser Met Leu Gly Met His Gly
    335                 340                 345

ACT GTT TAT GCT AAT TAT GCT GTG GAT AGT AGT GAT TTG TTG CTC GCA   1586
Thr Val Tyr Ala Asn Tyr Ala Val Asp Ser Ser Asp Leu Leu Leu Ala
350                 355                 360                 365

TTT GGG GTG AGG TTT GAT GAT AGA GTT ACT GGA AAG TTA GAA GCT TTT   1634
Phe Gly Val Arg Phe Asp Asp Arg Val Thr Gly Lys Leu Glu Ala Phe
                370                 375                 380

GCT AGC CGA GCG AAA ATT GTT CAC ATT GAT ATT GAT TCA GCC GAG ATT   1682
Ala Ser Arg Ala Lys Ile Val His Ile Asp Ile Asp Ser Ala Glu Ile
            385                 390                 395

GGA AAG AAC AAG CAG CCT CAT GTT TCC ATT TGT GCG GAT ATA AAG TTG   1730
Gly Lys Asn Lys Gln Pro His Val Ser Ile Cys Ala Asp Ile Lys Leu
        400                 405                 410
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GCA TTA CAG GGT TTG AAT TCG ATA TTG GAG AGT AAG GAA GGC AAA CTG   1778
Ala Leu Gln Gly Leu Asn Ser Ile Leu Glu Ser Lys Glu Gly Lys Leu
    415                 420                 425

AAG CTG GAT TTT TCT GCT TGG AGG CAG GAG TTG ACC GAG CAG AAA GTG   1826
Lys Leu Asp Phe Ser Ala Trp Arg Gln Glu Leu Thr Glu Gln Lys Val
430                 435                 440                 445

AAG TAC CCA TTG AAT TTT AAA ACT TTT GGT GAT GCT ATT CCT CCG CAA   1874
Lys Tyr Pro Leu Asn Phe Lys Thr Phe Gly Asp Ala Ile Pro Pro Gln
                450                 455                 460

TAT GCT ATC CAG GTT CTA GAT GAG TTA ACT AAT GGG AAT GCT ATT ATA   1922
Tyr Ala Ile Gln Val Leu Asp Glu Leu Thr Asn Gly Asn Ala Ile Ile
            465                 470                 475

AGT ACT GGA GTG GGT CAA CAC CAG ATG TGG GCT GCT CAA TAC TAT AAG   1970
Ser Thr Gly Val Gly Gln His Gln Met Trp Ala Ala Gln Tyr Tyr Lys
        480                 485                 490

TAC AGA AAG CCA CGC CAA TGG TTG ACG TCT GGT GGA TTA GGA GCG ATG   2018
Tyr Arg Lys Pro Arg Gln Trp Leu Thr Ser Gly Gly Leu Gly Ala Met
    495                 500                 505

GGA TTT GGT TTG CCC GCT GCT ATT GGT GCG GCT GTT GGA AGA CCG GAT   2066
Gly Phe Gly Leu Pro Ala Ala Ile Gly Ala Ala Val Gly Arg Pro Asp
510                 515                 520                 525

GAA ATT GTG GTT GAC ATT GAT GGC GAT GGC AGT TTC ATC ATG AAT GTG   2114
Glu Ile Val Val Asp Ile Asp Gly Asp Gly Ser Phe Ile Met Asn Val
                530                 535                 540

CAG GAG CTA GCA ACT ATT AAG GTG GAG AAT CTC CCA GTT AAG ATT ATG   2162
Gln Glu Leu Ala Thr Ile Lys Val Glu Asn Leu Pro Val Lys Ile Met
            545                 550                 555

TTA CTG AAT AAT CAA CAC TTG GGA ATG GTG GTT CAA TGG GAG GAT CGG   2210
Leu Leu Asn Asn Gln His Leu Gly Met Val Val Gln Trp Glu Asp Arg
        560                 565                 570

TTC TAC AAG GCT AAC AGA GCA CAC ACG TAC CTG GGG AAT CCT TCT AAT   2258
Phe Tyr Lys Ala Asn Arg Ala His Thr Tyr Leu Gly Asn Pro Ser Asn
    575                 580                 585

GAG GCG GAG ATC TTT CCT AAT ATG TTG AAA TTT GCA GAG GCT TGT GGC   2306
Glu Ala Glu Ile Phe Pro Asn Met Leu Lys Phe Ala Glu Ala Cys Gly
590                 595                 600                 605

ATA CCT GCT GCG AGA GTG ACA CAC AGG GAT GAT CTG AGA GCG GCT ATT   2354
Ile Pro Ala Ala Arg Val Thr His Arg Asp Asp Leu Arg Ala Ala Ile
                610                 615                 620

CAA AAG ATG TTA GAC ACT CCT GGG CCA TAC TTG TTG GAT GTG ATT GTA   2402
Gln Lys Met Leu Asp Thr Pro Gly Pro Tyr Leu Leu Asp Val Ile Val
            625                 630                 635

CCT CAT CAG GAA CAT GTT CTA CCT ATG ATT CCC AGT GGC GGG GCT TTC   2450
Pro His Gln Glu His Val Leu Pro Met Ile Pro Ser Gly Gly Ala Phe
        640                 645                 650

AAA GAT GTG ATC ACA GAG GGT GAC GGG AGA AGT TCC TAT TGA           2492
Lys Asp Val Ile Thr Glu Gly Asp Gly Arg Ser Ser Tyr  *
    655                 660                 665
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CTTTGAGAAG CTACAGAGCT AATTCTAGGC CTTGTATTAT CTAAAATAAA CTTCTATTAA 2552

GCCAAAGATG TTTTGTCTAT TAGTTTGTTA TTAGTTTTTG CCGTGGCTTT GCTCGTTGTC 2612

ACTGTTGTAC TATTAAATAG TTGATATTTA TGTTTGCTTT AAGTTTGCAT CAAAAAAAAA 2672

AAAAAAAAAA AAAAAAAAAA AAAACTCGAG 2702

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 4, line 16 - 17

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit	Accession Number
12 February 1997 (12.02.97)	97876

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

CLAIMS

What is claimed is:

1. An isolated nucleic acid fragment encoding the small subunit of a plant acetolactate synthase, said fragment comprising a member selected from the group

(a) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:4; and

(b) a nucleotide sequence essentially similar to the nucleotide sequence of (a).

2. The isolated nucleic acid fragment of Claim 1 wherein the nucleotide sequence is set forth in SEQ ID NO:4.

3. A plasmid vector comprising the nucleic acid fragment of Claim 1 operably linked to at least one suitable regulatory sequence.

4. A transformed host cell comprising the plasmid vector of Claim 3.

5. A method for evaluating at least one compound for its ability to inhibit acetolactate synthase activity, the method comprising:

(a) transforming a host cell with the plasmid vector of Claim 3;

(b) facilitating expression of the nucleic acid fragment encoding the small subunit of a plant acetolactate synthase;

(c) purifying the small subunit of a plant acetolactate synthase expressed by the transformed host cell;

(d) mixing the purified subunit of step (c) with the large subunit of a plant acetolactate synthase in a suitable container, thereby forming a plant acetolactate synthase holoenzyme;

(e) treating the holoenzyme from step (d) with a compound to be tested; and

(f) comparing the acetolactate synthase activity of the holoenzyme that has been treated with a test compound to the activity of an untreated holoenzyme,

and thereby selecting compounds with potential for herbicidal activity.

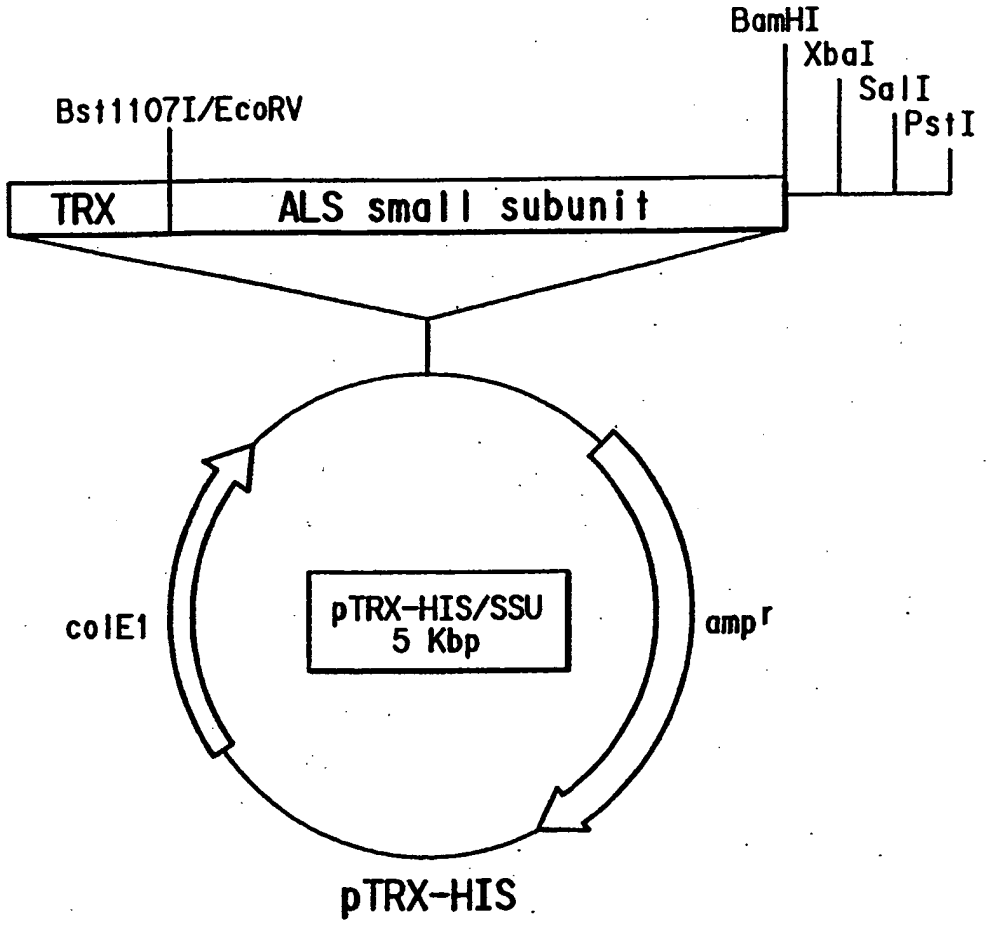
BamHI
XbaI
SalI
PstI
Bst1107I/EcoRV
TRX
ALS small subunit
colE1
pTRX-HIS/SSU
5 Kbp
ampr
pTRX-HIS

FIG.1

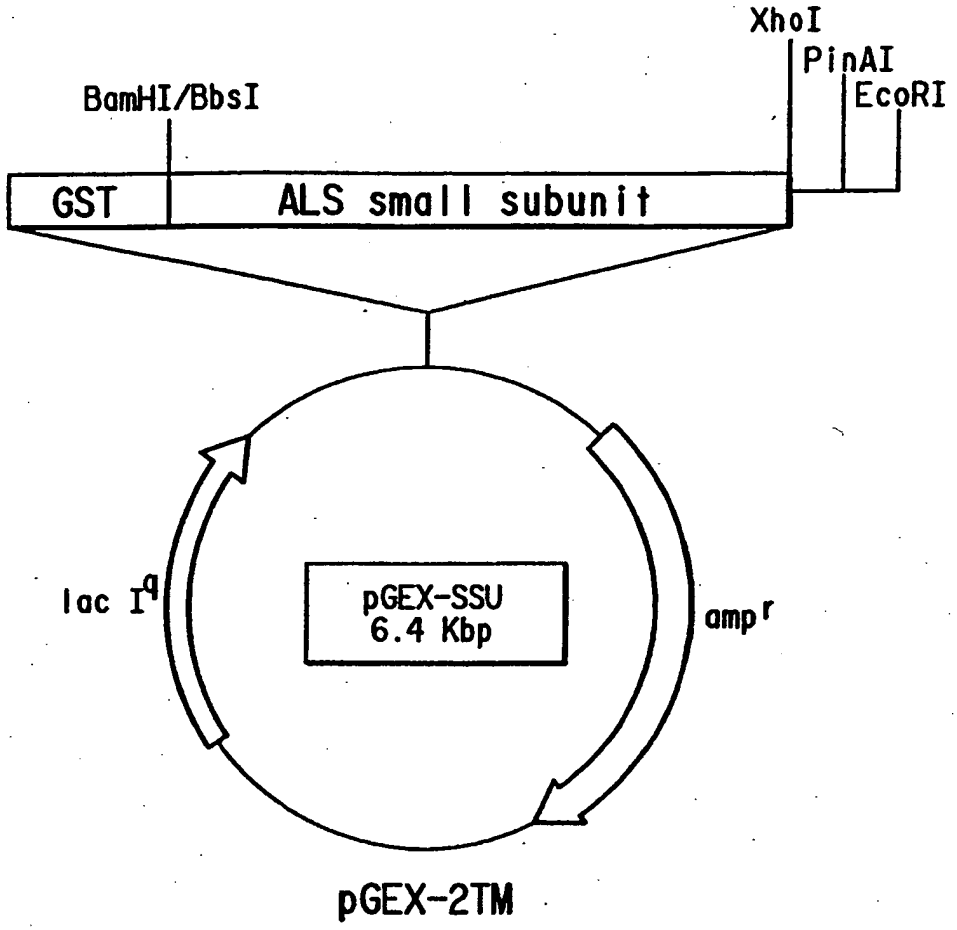
XhoI
PinAI
EcoRI
BamHI/BbsI
GST
ALS small subunit
pGEX-SSU
6.4 Kbp
lac I^q
amp^r
pGEX-2TM

FIG.2

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/03506

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/60 C12Q1/527

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 608 722 A (AMERICAN CYANAMID CO) 3 August 1994 see claim 4	5
A	US 5 356 789 A (SHANER DALE L) 18 October 1994 see claims	5

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 June 1998	16/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chakravarty, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intel ...nal Application No
PCT/US 98/03506

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0608722 A	03-08-1994	CA 2114357 A	30-07-1994
		JP 6253829 A	13-09-1994
US 5356789 A	18-10-1994	NONE	